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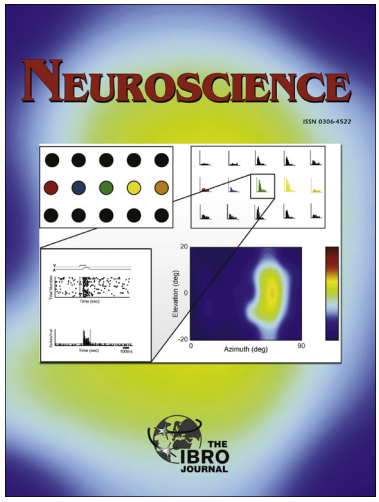

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Alterations in phospholipidomic profile in brain of mouse model of depression induced by chronic unpredictable stress

Renata Faria[1], Magda M. Santana[4,5], Célia A. Aveleira[5], Cláudia Simões[1], Elisabete Maciel[1], Tânia Melo[1], Deolinda Santinha[1], M Manuel Oliveira[2], Francisco Peixoto[3], Pedro Domingues[1], Cláudia Cavadas[4,5], M. Rosário M.Domingues[1]

[1] Mass Spectrometry Centre, UI-QOPNA, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

[2]CQVR, Chemistry Department, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

[3] CITAB, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

[4]Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal

[5]CNC - Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal,

Running title: Chronic unpredictable stress changes brain phospholipidome

*To whom correspondence should be addressed: Maria R. M. Domingues, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.

E-mail: mrd@ua.pt,

Telephone: + 351-234-370698, Fax: +351-234-370084

Abstract

Depression is a worldwide disability disease associated with high morbidity and has increased dramatically in the last few years. The differential diagnosis and the definition of an individualized therapy for Depression is hampered by the absence of specific biomarkers. The aim of this study was to evaluate the phospholipdiomic profile of brain and myocardium in a mouse model of depression induced by chronic unpredictable stress. The lipidomic profile was evaluated by thin layer and liquid chromatography and mass spectrometry and lipid oxidation was estimated by FOX II assay. Antioxidant enzymes activity and the GSH/GSSG ratio were also evaluated. Results showed that chronic stress affect primarily the lipid profile of the brain, inducing an increased in lipid hydroperoxides, which was not detected in the myocardium. A significant decrease in phospahtidylinositol (PI) and in cardiolipin (CL) relative contents and also oxidation of cardiolipin and significant increase of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were observed in in brain from mice after unpredictable chronic stress conditions. In myocardium only an increase in PC content was observed. Nevertheless, both organs present a decreased GSH/GSSG ratio when compared to control groups, corroborating the occurrence of oxidative stress. The enzyme activities CAT and SOD were found to be decreased in the myocardium and increased in the brain, while glutathione reductase (GR) was decreased in brain. Our results indicate that in a mouse model for studying depression induced by chronic unpredictable stress, the modification of the expression of oxidative stress related enzymes did not prevent lipid oxidation in organs, particularly in the brain. These observations suggest that depression has an impact in the brain lipidome and that further studies are needed to better understand lipid role in depression and to evaluate their potential as future biomarkers.

Keywords: Chronic stress, brain, phospholipids, mass spectrometry, lipidomic, oxidative stress

1. Introduction

Depression is a common and disabling psychiatric disorder with a lifetime prevalence of about 20%. Estimations from the World Health Organization predict that by the year of 2030 depression will be the second leading cause of disease burden worldwide (Joels et al., 2004, WHO, 2008, Huynh et al., 2011). Although the etiology of the disease remains to be completely understood, chronic stress has been considered one of the main factors that predispose the individuals to the development of depression (Krishnan and Nestler, 2008). During chronic stress, the hyperactivation of the sympathetic nervous system and a deregulation of the hypothalamus pituitary adrenal gland axis occur, leading to the increase of stress hormones, such catecholamine's and glucocorticoids (Chrousos, 2009, Hill et al., 2012). Up regulation of the stress hormones may induce the overproduction of free radicals and thus oxidative stress have been associated with depression progress (Michel et al., 2012). Oxidative stress induces changes in lipid profiling in consequence of lipid peroxidation, which have been associated with several neurological diseases. Also, the majority of lipid peroxidation products have pro inflammatory effects (Cohen, 2000, Black, 2002, Ahmad et al., 2010, Wielgat et al., 2011). Although, the pathophysiological alterations that triggers the association between chronic stress and depression are not completed understood, it is known that this association is characterized by an increase in cytokines/inflammation and oxidative stress with an increase in reactive oxygen species (Miller, 2010). Individuals with severe depression have shown inflammation, manifested by an increase of pro inflammatory cytokines levels, such as tumor necrosis factor (TNF-α), interleukins (IL-1 e IL-6), observed in

peripheral blood and cerebral spinal fluid, as well as an increased of the acute phase proteins, chemokines and adhesion molecules in peripheral blood (Miller, 2010).

Although chronic stress has been related with an increase in lipid peroxidation (Liu et al., 1996), its effect in brain lipid profile has not been investigated. The study of the physiological processes that occur in brain during depression, based on biochemical mechanisms associated to neuronal membrane modifications, reflect an increase in lipid peroxidation in every brain regions (Flerov and Gerasimova, 2007). This increase in lipid oxidation destabilizes membranes and may cause cell death (Tyurin et al., 2009).

The chronic unpredictable stress (CUS) paradigm is a widely used rodent model of depression, originally described by Katz and Hersh (Katz and Hersh, 1981) and further developed by Willner et al (Willner et al., 1987), which emphasizes the role of stress in the etiology of depression. In this model, animals gradually develop a chronic depressive-like state due to sequential and unpredictable exposures to different stressors, over a sustained period of time. CUS induces long lasting changes of behavioral and neurobiological parameters resembling dysfunctions observed in depressed patients (Willner, 1997, 2005, Hill et al., 2012).

The aim of this study is to describe the alterations on phospholipid profile in brain and myocardium in a mouse model of depression induced by chronic unpredictable stress. The presence of oxidative stress promoted by CUS was validated by the GSH/GSSG ratio and the observation of lipid peroxidation. Some key enzymes against oxidative stress were also measured. The lipid profile was evaluated using a lipidomic approach: The different phospholipid classes were initially separated by TLC and quantified by phosphorous assay. The total lipid extracts from brain and myocardium were then analyzed by HPLC-MS and MS/MS.

2. Materials and Methods

Chemicals

The phosphatidylethanolamine (PE - 14:0/14:0), phosphatidylserine (PS - 14:0/14:0), phosphatidylcholine (PC -14:0/14:0), phosphatidylinositol (PI - 16:0/16:0), phosphatidic acid (PA - 14:0/14:0), sphingomyelin (SM - d18:1/18:1), phosphatidylglycerol (PG - 14:0/14:0), lysophosphatdylcholine (LPC - 18:0), lysophosphatidylinositol (LPI - 18:0), phosphatidylethanolamine (LPE - 18:0), lysophosphatidic acid (LPA - 18:0) and cardiolipin (CL - 14:0/14:0/14:0/14:0) standards were purchased from Sigma-Aldrich (Madrid, Spain), triethylamine (Acros Organics), chloroform (HPLC grades), methanol (HPLC grades), ethanol (Panreac), primuline (Sigma) were used without further purification. TLC silica gel 60 plates with concentration zone (2.5x20cm) were purchased from Merck (Darmstadt, Germany).

Animals

Male, 9-weeks old C57/BL6 mice (Charles River, Barcelona) were individually housed under a 12h light/dark cycle in a humidity/temperature controlled room, with *ad libitum* access to a standard chow diet and water, except when food and water deprivation was specified by the stress protocol. Animals were allowed 5 days to acclimatize to the surroundings before each stress protocol. All experimental procedures were performed in accordance with the European Union Directive 86/609/EEC for the care and use of laboratory animals. All people working with animals have received appropriate education (FELASA course) as required by the Portuguese authorities. In addition, animals are housed in our licensed animal facility

(International Animal Welfare Assurance number 520.000.000.2006). The present study and the animal experimentation described were included in a project approved and financed by the Portuguese Science Foundation. Center for Neuroscience and Cell Biology (CNC) animal experimentation board also approved the utilization of animals for this project (reference PTDC/SAU-FCF/108110/2008).

Chronic unpredictable stress protocol

The chronic unpredictable stress protocol was performed as previously described by Willner P (Willner et al., 1987), with some modifications. Mice were exposed to different stressors that were applied once a day, during a period of 21 days, and in the following order: day 1 - exposure to the box with wet shavings (24h); day 2 - pairing with another stressed mice (1h); day 3 - cold bath (15ºC, 20 min); day 4 - enclosure in a tube (2h); day 5 - foot shock (0.7mA, 3s, given intermittently during a total time of 5min); day 6 - exposure to the apparatus of foot shock without the shocks (1h); day 7 - light off in the light phase of the cycle and box without shavings (24h); day 8 - inclined box (45º, 24h); day 9 - deprivation of water and food (24h); day 10 - access to the empty bottle (1h); day 11 - exposure to the box with wet shavings (24h); day 12 - pairing with another stressed mice (1h); day 13 - cold bath (15 ºC, 20 min); day 14 - enclosure in a tube (3h); day 15 - foot shock (0.7mA, 3s, given intermittently during a total time of 5min); day 16 - exposure to the apparatus of foot shock without the shocks (1h); day 17 - deprivation of water and food (24h); day 18 - access to the empty bottle (1h); day 19 - enclosure in a tube (4h); day 20 - light off in the light phase of the cycle and box without shavings (24h); day 21 - inclined box (45º, 24h). Animals were used for behavioural tests or sacrificed for tissue collection 24h after the last stressor.

Forced swimming test

The forced swimming test (FST) was performed to evaluate depressive behavior of mice (Cryan and Mombereau, 2004, David and John, 2012). Mice were dropped individually into glass cylinders (height: 25 cm, internal diameter: 10 cm) containing 15 cm water, maintained at 23-25ºC, and the immobility time (ceased struggling and remained floating motionless in the water) for a 6-min period was measured. An increase in immobility over time is considered to be associated with behavioral despair, which is a feature of a depressive state (Cryan and Mombereau, 2004, David and John, 2012).

Tissue collection

Mice were anesthetized and killed by decapitation. The myocardium and brain were removed and immediately frozen on dry ice for further phospholipidomic study.

Determination of antioxidant enzymes

Myocardium and brain mice were weighed and added to 10% (w/v) 50 mM phosphate buffer, pH 7,0 and were homogenized. The homogenates obtained were centrifuged at 16,000 xg for 20 min at 4ºC, the protein supernatant was quantified by the procedure of Bradford (Bradford, 1976), calibrated with BSA standards. This supernatant was used to measure antioxidant enzyme activities and the concentration of reduced (GSH) and oxidized (GSSG) glutathione. Superoxide dismutase (SOD) activity was assayed as previously described (Oliveira et al., 2013). Catalase (CAT) activity was assayed polarographically using a Clark-type oxygen electrode (Hansatech Instruments Ltd.). Assays were conducted as previously described

(Oliveira et al. 2013). Glutathione reductase (GR) activity was performed according to Smith et al. (Smith et al., 1988).

Measurement of oxidized/reduced glutathione ratio

Concentrations of GSH and GSSG were measured from the supernatant fraction that was obtained in the previously described enzyme assays after ultrasonication and centrifugation. The supernatant, kept at 0ºC, was used for the GSH and GSSG assays on the same day. This determination was done by spectrofluorimetry, as previously described by Galhano et al. (Galhano et al., 2010). .

Protein Quantification

Protein quantification was determined with DC Protein Assay (BioRad, Hercules, CA, USA. For protein quantification)we added 20 µL of the reagent S (surfactant solution) to 1mL of reagent A (an alkaline cooper tartrate solution), to do reagent A'. Standards of BSA 0.2 mg/mL to 1.5 mg/mL were also prepared. Then, 5 µL of each standard were mixed and each sample with 25 µL of reagent A' and 200 µL of reagent B (a dilute Folin Reagent), and incubated at room temperature 15min. The absorbance at 750 nm was read using a microplate reader (Multiscan 90, ThermoScientific).

Phospholipid Extraction

Brain and myocardium samples were homogenized with 1 mL potassium phosphate buffer (50 mM, pH 7.0). Phospholipids were extracted from the brain and from the myocardium of mice by the Bligh and Dyer method (Bligh and Dyer, 1959).

Briefly, 3.75mL of $CHCl_3$: MeOH (1:2 v/v) were added to 1 mL of sample (homogenized tissue), well mixed in a vortex and incubated in ice for 30 min. Then, 1.25 mL of $CHCl_3$ and 1.25 mL of H_2O were added and mixed well. After, the mixture was centrifuged at 1,000 x g, 5 min, at room temperature (centrifuge Mixtasel Centrifuge (Selecta)), obtaining two phases: the aqueous phase on top and the organic phase below, from where lipids were collected. The extracts were dry under nitrogen stream and stored at -20 ºC, for subsequent analysis.

Quantification of Phospholipid classes using phosphorous assay

In order to determine the content of phospholipids of each extract and compare with the phospholipid content of each class separated by TLC, a phosphorous quantification method was realized in accordance with the protocol of Bartlett and Lewis (Bartlett and Lewis, 1970). In brief, 650 µL of percloric acid (70% m/v) were added to the samples, which were incubated 45 min at 180ºC in a heating bloc (Stuart, U.K.). To all samples we added 3.3 mL of H_2O, 0.5 mL of ammonium molybdate (2.5 g ammonium molybdate/100 mL of H_2O) and 0.5 mL of ascorbic acid (10 g ascorbic acid / 100 mL of H_2O), and vortexed after the addition of each solution, following by the incubation in a bath at 100ºC, 5 min. Additionally we prepared standards with 0.1 to 2 µg of phosphate, which suffer the same treatment of the samples. Finally, we measured the absorbance of standards and samples at 800 nm, in a microplate reader (Multiscan 90, ThermoScientific). The percentage of each phospholipid class was calculated, relative to the total amount of phosphorous in the sample, thus giving the relative abundance of each phospholipid class.

Quantification of lipid hydroperoxides using FOX II assay

In order to quantify lipid hydroperoxides, 50 µL of the total lipid extract was added to 950 µL of the FOX reagent solution (100 µM xylenol orange, 250 µM Fe^{2+}, 25 mM H_2SO_4, and 4 mM BHT in 90% (v/v) methanol) in microtubes, homogenized in a vortex mixer and incubated for 30 min at room temperature, in the dark. After incubation, the absorbance of samples was read at 560 nm against H_2O_2 standards with concentrations ranging from 0.0-0.4 mM (H_2O_2 1 mM, FOX2 reagent and water). The FOX reagent (100 mL) was prepared as follows: 250 µM $(NH_4)2Fe(SO_4).6H_2O$ (9.8 mg) and 25 mM H_2SO_4 (139 µL) were dissolved in 5 mL of water, mixed with 4 mM 2,6-di-tert-butyl-p-hydroxytoluene (BHT) (88.2 mg), 100 µM xylenol orange (7.2 mg) and 45 mL of methanol. Then, other 45 mL of methanol and 5 mL of water were added (Jiang et al., 1991).

Separation of Phospholipid Classes by Thin layer chromatography

Separation of PL classes by TLC from the total lipid extract was performed using plates of silica gel 60 with concentration zone 2.5x20 cm. Before TLC separation, the plates were washed in CHCl3: MeOH (1:1 v/v) and treated with boric acid in ethanol (2.3% w/v). The plates with spots containing about 30 µg of sample were developed in a mixture of solvents $CHCl_3$: MeOH: H_2O: trietilamine (30:35:7:35, v/v/v/v). To reveal the phospholipid spots, the TLC plates were sprinkle with a primuline solution (50 µg/100 mL acetone: water, 80/20, v/v), and visualized with UV lamp (λ=254nm). After the identification of the phospholipid spots, by comparison with phospholipid standards, the spots were scraped off from the plates and quantified using the phosphorous assay.

HPLC conditions

In order to identify the molecular species and their changes in chronic stress, phospholipid classes were separated by HILIC-LC-MS, using a HPLC system (Waters Alliance 2690) coupled to an electrospray (ESI) linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). The mobile phase A consisted of 10% water and 55% acetonitrile with 35% (v/v) of methanol. The mobile phase B consisted of acetonitrile 60%, methanol 40% with 10mM ammonium acetate. 15 µL of total lipid extract were diluted in the mobile phase B and reaction mixture was introduced into a Ascentis Si HPLC Pore column (15 cm×1.0 mm, 3 µm) (Sigma-Aldrich). The solvent gradient was programmed as follows: gradient started with 0% of A and linear increased to 100% of A during 20 min, and held isocratically for 35 min, returning, to the initial conditions in 5 min. The flow rate through the column was 16 µL/min obtained using a pre-column split (Acurate, LC Packings, USA). LC-MS was performed with an internal standard to confirm and quantify the ions variations observed in the spectrum according to the Lipid Maps methods (Ivanova et al., 2009). The PL standards used were PC (14:0/14:0), PS (14:0/14:0), PI (16:0/16:0), PE (14:0/14:0), PA (14:0/14:0) and CL (14:0/14:0/14:0/14:0).

Electrospray mass spectrometry conditions

The phospholipid analysis was carried out in positive and negative mode on electrospray (ESI) linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). ESI conditions on linear ion trap mass spectrometer were as follows: electrospray voltage was 4.7 kV in negative mode and 5 kV in positive mode; capillary temperature was 275ºC and the sheath gas flow was 25 units. An isolation width of 0.5 Da was used with a 30 ms activation time for MS/MS experiments. Full

scan mass spectrum and MS/MS spectrum were acquired with a 50 ms and 200 ms maximum ionization time, respectively. Normalized collision energyTM (CE) was varied between 17 and 20 (arbitrary units) for MS/MS. Data acquisition and treatment of results was carried out with an Xcalibur data system (V2.0).

Statistical Analysis

Results were presented as means ± standard error (SD). Differences among experimental groups were determined by unpaired t-test. P-values <0.05 were considered statistically significant.

3. Results

Forced swimming test (FST) and adrenal gland weight

The FST was performed to evaluate the stress protocol. After 21 days of exposure to unpredictable stress, mice show higher immobility time (235.6±15.9s; mean±SEM, n=5) compared with the control group (140.8±3.8s, mean±SEM, n=5, $p<0.001$) in FST. In addition, adrenal glands of stressed mice were significantly heavier than control mice adrenals (control=1.72±0.05mg; stressed mice=2.06±0.08mg; mean±SEM; n=6; $p<0.01$).

Enzymatic oxidative stress and GSH/GSSG ratio

The results obtained for the evaluation of the oxidative stress enzymes were summarized in Table 1. Catalase activity (CAT) was significantly decreased (25.4%, $p<0.0001$) in rat myocardium submitted to chronic unpredictable stress, when compared to control group. Curiously, brain obtained from mice after stress stimuli

showed an increase (80%) in catalase activity that was significantly different ($p<0.0064$) when compared with the control group (Table 1). The superoxide dismutase (SOD) measured in brain showed a significant difference when compared to control group (Table 1) while no significant difference was found for glutathione reductase (GR). We also evaluated the GSH/GSSG ratio since it is considered to be a sensitive indicator of the cellular redox state. Data obtained in myocardium of mice submitted to chronic stress showed a decrease (37%) in GSH/GSSG ratio statistically significant ($p=0.032$). In mice brain of stressed group the decrease of GSH/GSSG ratio (44%) was also significantly different from the control group ($p=0.002$).

Quantification of lipid hydroperoxides in mice brain and myocardium after exposure to chronic stress

Some authors have reported the increase of oxidative stress, under chronic stress conditions (Liu et al., 1996, Lucca et al., 2009) . Lipid hydroperoxides are the primary products of lipid oxidation and have been used in several studies to evaluate the oxidation status of cells and/or tissues (Tyurin et al., 2008, Sparvero et al., 2010). In this work we have assessed lipid hydroperoxides using FOX II assay. Results showed a statistically significant increase of lipid hydroperoxides in the total lipid extracts from brain of the chronic stress group (Figure 1A), indicating that lipid oxidation occurred in consequence of the stress stimuli. Interestingly, no significant increase in lipid hydroperoxides was observed in the lipid extract obtained from heart of mice after stress (Figure 1B). Lipid peroxidation is a reaction that causes many changes in the homeostasis of the organism, since it is involved in cellular damage and may explain the neuronal loss verified in stress situations. This phenomenon is

also involved in tissue damage, aging and neurodegenerative diseases (Liu et al., 1996).

Effects of chronic stress in lipid profiling of brain

Total protein amount of brain tissues and total phospholipid content obtained in the total lipid extract was determined in the brain of control and stressed mice (Table S1). No statistically significant differences were observed in the amount of protein per mg of tissue and in the PL content per mg of tissue. However, the ratio of the PL and protein content showed a tendency to increase in mice after chronic stress. These observations suggest that the changes in phospholipid metabolism and profilie may occurred in a response to the stress stimuli.

In order to evaluate alterations in the PL profile of mice brain after stress stimuli, the total extracted PLs were fractionated by thin layer chromatography (TLC), (Figure 2). Each class was identified by comparison with pure phospholipid standards applied in the same TLC plate. Relative abundance of each PL class observed in the TLC plate was determined by quantification of phosphate content determined in each PL spot (Figure 2). This analysis was carried out in triplicate for each sample and three different samples were analyzed for each experimental group. This approach allowed the separation of seven brain phospholipid classes: sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidic acid (PA) and cardiolipin (CL) (Figure 2). The most abundant PL class was PC, followed by PE. PS, PI, CL, SM and PA (Figure 2).

Differences in the relative contents in brain phospholipid profile of chronic stressed mice were observed for PC, PE, PI and CL (Figure 2). The exposure to

chronic stress caused a significant increase in PC and PE levels, while phosphatidylinositol and cardiolipin classes presented a very significant decrease (Figure 2) No significant changes were detected for SM, PS and PA content between control and chronic stress group.

In order to evaluate alterations in the molecular composition of each PL class analysis by HPLC-MS was performed). PC, SM and LPC classes were analyzed by LC-MS in the positive mode, while PE, PA, PS, PI e CL were analyzed by LC-MS in the negative mode (Schwalbe-Herrmann et al., 2010). LC-MS and MS/MS interpretation for each ion, allowed identification of PL molecular species. Similar molecular profile was observed for all classes except for CL .

Due to some co-elution of PI with CL, the CL profile was also analyzed after TLC separation. The ESI-MS spectra obtained are shown in Figure 3. The most significant differences observed correspond to the decrease in relative abundance of the CL $[M-2H]^{2-}$ ions at *m/z* 723.4, 737.5, 749.6 and 751.8 and the increase of ions at *m/z* 697.8, 761.7, 773.6 in the MS spectrum obtained from mice brain under chronic stress (Table 2). The increase of $[M-2H]^{2-}$ ions at *m/z* 697.8, 761.7, 773.6 were attribute to the formation of new CL oxidation products. The ESI-MS/MS spectra of these new ions allowed the confirmation of the formation of these new CL oxidation species and alloed also to pinpoint the oxidative modifications in unsaturated fatty acyl chains. These CL oxidation products were identified as short (at *m/z* 697.8) and long-chain oxidation products of CL (at *m/z* 761.7, 773.6).

Short chain oxidation products of CL, were observed previously *in vitro* and *in vivo* and characterized by MS/MS analysis (Maciel et al., 2011). Figure 4 shows the MS/MS spectrum of the ion at *m/z* 697.8, which analysis and interpretation allowed the assignment of this ion as the short chain oxidation product CL-

$(C18:2)_3(C10:2(O)COOH)$. The product ion at *m/z* 1167, arised from the loss of shortened fatty acyl chain (HOOC10:2(O)COOH, - 228 Da) and the carboxylate anion ($RCOO^-$) of non-modified linoleic acid at *m/z* 279.1 supported the previous identification. This oxidation product can result from oxidative cleavage of the C11-C12 bond, in the unsaturated C20:4, C20:3 or C22:6 fatty acyl chain of different CL species. Thus, this short chain product may result from oxidation of CL-$(C18:2)_3$(C20:4) (*m/z* 735.5), CL-$(C18:2)_3$(20:3) (*m/z* 736.5) or CL-$(C18:2)_3$(C22:6) (*m/z* 747.5).

The CL hydroperoxy and hydroxy derivatives were already observed in CL oxidation in *in vitro* and *in vivo* studies (Tyurin et al., 2000, Tyurina et al., 2011). In this study, we have identified hydroxy and hydroperoxy –CL species at *m/z* 761.7 and 773.6 (Table 2). The CL oxidation species observed at m/z 761.7 has presumably been formed by oxidative modification of oleoyl fatty acyl chain (+3O) from the CL specie observed at *m/z* 737.5, whose relative abundance decrease in the MS spectrum after chronic stress stimuli. The MS/MS spectrum of the ion at *m/z* 761.7 (Figure 4) showed the carboxylate anions at *m/z* 255 (C16:0), 327 (C22:6), 297 (C18:1+O) and 295 (C18:1+2O-H_2O). Loss of water from hydroperoxides during the MS/MS fragmentation is a very common process (Kim et al., 2011).

The observed increase of the relative abundance of the ion at *m/z* 773.6 may be due to oxidation of the CL species bearing oleoyl and linoleoyl fatty acyl chains, identified as $[M-2H]^{2-}$ at *m/z* 749.6, whose relative abundance also decreased in the MS spectrum, obtained after chronic stress stimuli. The analysis of MS/MS spectrum (Figure 4) allowed the identification of three CL oxidation species (resumed in table 2) by the presence of carboxylate anion at *m/z* 313 (C18:1+2O), 311 (C18:2+2O) and 295 (C18:2+O or/and C18:1+2O-H_2O)..

Effects of chronic stress in lipid profiling in myocardium

The relationship between the total amount of phospholipid and the concentration of protein from myocardium did not show differences statistically significant (Table S1). TLC analysis and phosphorous quantification of the total lipid extract from myocardium of mice control and after stress allowed (Figure 5) to disclose only a significant increase of the PC relative content. No significant increase in lipid hydroperoxides was observed in myocardium after chronic stress stimuli (Figure 1B). It is important to notice that myocardium is less susceptible to oxidation than brain, since it contains PLs with less unsaturated fatty acids when compared with brain lipids, which are specially rich in polyunsaturated fatty acids. The analysis of the phospholipid classes by HPLC-MS and HPLC-MS/MS did not revealed any significant differences in PL molecular composition.

4. Discussion

Chronic stress has been related with the development of several pathophysiological disturbances, due to the deregulation of many biochemical and physical processes (Paul H, 2002). The close relationship between chronic stress and depression has drawn much attention in the scientific community, since depression is a major socioeconomic burden of disease. There is wide consensus and support from many different studies that long-term exposures to life stressors play a key role in the development of depression. Chronic stress has a stronger association with depressive episodes and also predicts higher levels and amplifies the impact of acute stress events on the onset of depressive states (McGonagle and Kessler, 1990, Kessler, 1997, Paykel, 2003, Hammen et al., 2009).

The CUS model of depression was originally performed by Katz and Hersh (Katz and Hersh, 1981) and further developed by Willner et al (Willner et al., 1987) to induce a chronic depressive-like state in rodents, as a consequence of unpredictable and sequentially exposures to a variety of stressors over a sustained period of time. The validity and reliability of CUS as a model of depression have already been described (Willner, 1997, 2005, Hill et al., 2012).

In agreement with previous studies, in the present work, mice submitted to 21 days of CUS developed a depressive-like behavior, as it was demonstrated by the increased immobility time during FST. Increased immobility following CUS was seen in animals showing decreased reactivity to rewards and has been associated with an anhedonic state (Cryan and Mombereau, 2004, Strekalova et al., 2004, David and John, 2012). An enlargement of adrenal gland is also often described in chronic stressed mice (Scaria and Premalatha, 1967, Gosney, 1985, Mitchell and Vulliet, 1987, Ulrich-Lai et al., 2006). As expected, an increase in adrenal gland weight was observed after 21 days of CUS.

Chronic stress has been correlated with oxidative stress and increased production of ROS (Zafir and Banu, 2009). Moreover, several studies have correlated the biochemical changes occurred in central nervous system with impaired brain function and its consequences in myocardium diseases (Black and Garbutt, 2002, Pereira et al., 2012). In both of these organs, phospholipids are very important biomolecules and their profile alterations have been related with many diseases of the brain and of the myocardium (Han, 2010, Kosicek M and Hecimovic S 2013, Salomon 2012). Therefore, we examined the effects of CUS on superoxide dismutase, catalase (CAT), glutathione reductase activities and glutathione oxidation in mice brain and myocardium.

In the present study, the CAT activity was found to be significantly decreased in myocardium of stressed animals while it was increased in the brain. Kaushik and Kaur (Kaushik and Kaur, 2003) also found a significant decrease in CAT activity on myocardium of cold stressed animals, but no alterations on CAT activity were observed in brain. These results are apparently contradictory with those obtained by Sahin and Gumuslu (Sahin and Gumuslu, 2004b, a) since they have found that in rat brain this type of stress induced an increase in CAT and SOD, which is in agreement with our results. Several studies have shown that chronic stress exposure could induce different alterations in oxidative status that are dependent on the tissue under study but also on the type of stress (Kaushik and Kaur, 2003, Sahin and Gumuslu, 2004b, a). Therefore, the apparent lack of consistency in the antioxidant enzymes activities obtained by different authors could result from differences in protocols used for induction of stress as well as the animal models used.

The oxidation of glutathione and the protein-bound accessible thiols are considered one of the first and most important events that leads to a change in the overall cellular redox state (Giles et al., 2003). Thus, the GSH/GSSG ratio is considered to be a sensitive indicator of the cellular redox state (Jones, 2002). It has already been shown that chronic stress causes a decrease in the levels of glutathione, leading to a decrease in the GSH/GSSG (Madrigal et al., 2001, Samson et al., 2005). The present results allow us to validate the presence of oxidative stress in this model of chronic stress since the GSH/GSSG ratio decreased in this group, both in myocardium and brain. The increase in lipid hydroperoxides and GSH/GSSG ratio in brain, suggest an increase in ROS production.

A lipidomic approach was used to evaluate the effects of chronic unpredictable stress in phospholipids profile of brain and myocardium from chronic

stressed mice. Chronic unpredictable stress and depression induced changes in relative content of each PL class in brain and in plasma, probably due to a change in the PL metabolism.(Demirkan A, et al,2013) Also, oxidation of cardiolipin was observed. On the other hand, no significant changes were observed in the relative content of PL classes in the myocardium, except in PC class that showed an increase. The increase in PC observed may suggest a protective response to stress, since higher content of PC seems to improve the myocardial function (Van de Velde et al., 2000). However, a similar tendency in the variation of the relative content of each phospholipid class, suggests the possibility that the changes found in brain may occur also in myocardium, when the chronic stress stimulus is longer. Thus, this may suggest that stress has an initial impact in brain and probably the cardiovascular system is affected later on.

The alterations observed in PL profile in the brain were mainly related to changes in CL and PI. It was observed a decrease in CL relative content and, simultaneously, an increased CL oxidation together with a decrease in the PI relative content. CL is an exclusive phospholipid of mitochondria. ROS formation occurs preferentially in mitochondria, and therefore it is accepted that CL is more susceptible to oxidation than other phospholipids. Moreover, because of the structural diversity of CL in brain, detecting hydroperoxide species of CL by MS is challenging, since CL oxidized species are likely to be overlap the natural brain CL species (Sparvero et al., 2010). In addition to the observed oxidation of CL, we also reported a decrease in the total content of CL after chronic unpredictable stress, which might be implicated in a decline of mitochondrial respiratory functions and ROS accumulation. It is clear that during cell death , the increase of ROS and the loss of CL are closely linked in a cycle of CL peroxidation (Gonzalvez and Gottlieb, 2007). Several studies indicate that

peroxidized CL is unable to support the reconstituted activity of mitochondrial respiratory enzymes. Furthermore, CL peroxidation may lead to an overall loss of detectable CL content, either by preferential hydrolysis of peroxidized acyl chains by PLA_2, direct decomposition of lipid peroxides, or the formation of CL- protein complexes (Chicco and Sparagna, 2007). Moreover, the loss of CL content and CL oxidation are related with the development of many diseases .Several studies have correlated oxidative changes in CL with neuronal diseases and brain injuries (Adibhatla et al., 2006, Adibhatla and Hatcher, 2007, Bayir et al., 2007, Adibhatla and Hatcher, 2008, Tyurin et al., 2008, Tyurin et al., 2009). The reduction of CL level in the brain with aging has been reported and associated with an increase of lipid peroxidation in rat brain mitochondria exposed to oxidative stress (Paradies et al., 2011). Thus, the CL oxidation observed in our study might suggest a functional impairment of mitochondria after chronic unpredictable stress. The observed brain CL oxidation together with the decrease in CL content can be associated with neuronal loss or dysfunction in consequence of chronic stress, thus underlying the physiopathological process of depression, similarly as reported with the development of neurodegenerative diseases (Pope et al., 2008).

PIs are a group of phospholipids widely distributed in nature and which are involved in secretory events and in intercellular signaling. The metabolism of inositol lipids is involved in the signal transduction of many hormones, neurotransmitters and growth factors. PI classes are precursors of important signaling molecules that modulate cell growth, proliferation and death (Wymann and Schneiter, 2008). In the present study, the significant decrease in PI class in brain observed after chronic unpredictable stress suggests changes in cellular signaling processes in response to stress stimuli. Despite the difference in the relative content of the PI class in the two

study groups, the PI profiles are similar. PIs can be phosphorylated with formation of phosphorylated phosphoinositides (PIPs), which have various functions depending on the position of phosphorylation in the inositol head group. These molecules are extremely important since they are responsible for triggering signaling cascades, playing a main role in cell metabolism and apoptotic cellular events (Hicks et al., 2006). Changes in lipid metabolism may be due to an increase in phospholipase C action, a phospholipase specific for PI class. Phospholipase C enzymes hydrolyze PIP2 to generate the hydrophilic acidic end-group inositol(1,4,5)trisphosphate (I (1,4,5)P3) and the neutral lipid sn-1,2-diacylglycerol (DAG) (Woodcock et al., 2009). I(1,4,5)P_3 mobilize intracellular Ca^{2+} causing the depolarization of neuronal membrane; the other product DAG is an activator of protein kinase C, which accumulation has been associated with neurodegenerative diseases (Adibhatla et al., 2006).

Conclusions

In the present study, we reported that chronic unpredictable stress exposures in mice are accompanied by significant changes in the cellular levels of both signaling and structural lipids from brain, due to the increase in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) contents and a decrease in phosphatidylinositol (PI), phosphatidic acid (PA) and cardiolipin (CL) contents. Lipid profiling of myocardium was almost not affected and only showed a significant increase in the PC class. However, it was possible to identify cardiolipin hydroperoxides in brain lipid extracts after chronic stress conditions, indicating that the increase in hydroperoxy content was due to CL oxidation. Interestingly CL oxidation is an early indicator of cell apoptosis that could be associated with neuronal death. Results from FOX assay

indicate that lipid peroxidation occur in brain, but not in myocardium. Nevertheless, both organs present a diminished GSH/GSSG ratio compared to control groups that indicate the occurrence of oxidative stress. With regard to the enzymes activities CAT and SOD decreased in myocardium an augmented in brain, while GR had an inverse behavior. In the present model of depression, the observed alterations on the enzymes of oxidative stress were not sufficient to prevent the oxidation of cell components. Further studies are necessary to explore the functional relevance of the changes in lipid profiling occurring during chronic unpredictable stress exposures and their impact on depression development and prognosis.

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CAPTIONS

Figure 1. Concentration of lipid hydroperoxides in lipid extracts obtained from A) brain and B) myocardium of mice samples, from control group (CTL) and chronic stress group (Stress), evaluated by FOX II assay. * $p < 0.05$ versus control; n=3 independent experiments.

Figure 2. Thin-layer chromatography of total lipid extract obtained from mice brain control (CTL) and with chronic stress (Stress). Phospholipid standards were also applied: (PC) - Phosphatidylcholine; (PS) - Phosphatidylserine; (PE) - Phosphatidylethanolamine; (SM) - Sphingomyelin; (PG) - Phosphatidylglycerol; (LPI) - Lysophosphatdylinositol; (LPE) - Lysophosphatidylethanolamine; (PI) - Phosphatidylinositol; (PA) - Phosphatidic Acid; (CL) - Cardiolipin; (LPC) - Lysophosphatidylcholine; (LPA) - Lysophosphatidic acid. Relative content of phospholipid classes in controls (CTL) and chronic stress situations (Stress) in total lipid extract obtained from mice brain. The phospholipid classes were separated by thin-layer chromatography and the phosphorous content of each spot was calculated taking in account the amount of phosphorous in the total lipid extract. Phospholipid classes were separated and quantified: (SM) - Sphingomyelin; (PC) - Phosphatidylcholine; (PI) - Phosphatidylinositol; (PS) - Phosphatidylserine; (PE) - Phosphatidylethanolamine; (PA) - Phosphatidic Acid; (CL) - Cardiolipin. * $p < 0.05$ versus control, ** $p < 0.01$ versus control, n=3 independent experiments.

Figure 3. MS spectra of CL obtain from brain of mice control (A) and from brain of mice after chronic stress (B). *Impurities

Figure 4: Tandem mass spectrometry analysis of CL oxidation products identified in mice brain with chronic stress. MS/MS spectrum of the ion $[M-2H]^{2-}$ at m/z 697,

corresponding a short chain oxidation product with a carboxylic group terminal (A). MS/MS spectrum of the ion $[M-2H]^{2-}$ at m/z 761, corresponding hydroperoxy and hydroxy CL derivatives (B). MS/MS spectrum of the ion $[M-2H]^{2-}$ at m/z 773, corresponding hydroperoxy and hydroxy CL derivatives (B).

Figure 5. Thin-layer chromatography of total lipid extract obtained from mice myocardium control (CTL) and with chronic stress (Stress). Phospholipid standards were also applied: (PC) - Phosphatidylcholine; (PS) - Phosphatidylserine; (PE) - Phosphatidylethanolamine; (SM) - Sphingomyelin; (PG) - Phosphatidylglycerol; (LPI) - Lysophosphatdylinositol; (LPE) - Lysophosphatidylethanolamine; (PI) - Phosphatidylinositol; (PA) - Phosphatidic Acid; (CL) - Cardiolipin. Relative abundance of phospholipids, in controls (CTL) and chronic stress situations (Stress), from mice myocardium. The phospholipid classes were separated by thin-layer chromatography and the phosphorous content of each spot was calculated taking in account the amount of phosphorous in the total lipid extract. In this case we were able to separate eight different classes and quantified them: (LPC) - Lysophosphatidylcholine; (SM) - Sphingomyelin; (PC) - Phosphatidylcholine; (PI) - Phosphatidylinositol; (PS) - Phosphatidylserine; (PG) - Phosphatidyglycerol; (PE) - Phosphatidylethanolamine; (CL) - Cardiolipin. *$p < 0.05$ versus control, n=3 independent experiments.

Table 1. Effect of chronic unpredictable stress on antioxidant enzymes and GSH/GSSG ratio from myocardium and brain mice.

Table 2. Main decrease CL species and oxidized species identified in CL spectra obtained from mice brain under chronic stress conditions.

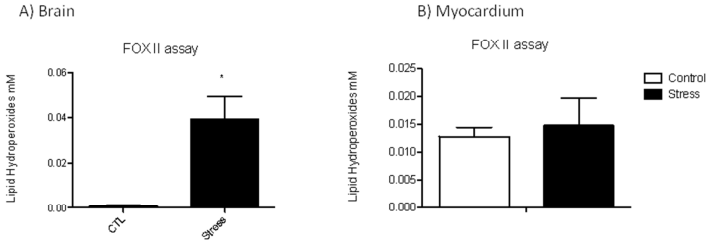
Table 1. Effect of chronic unpredictable stress on antioxidant enzymes and GSH/GSSG ratio from myocardium and brain mice

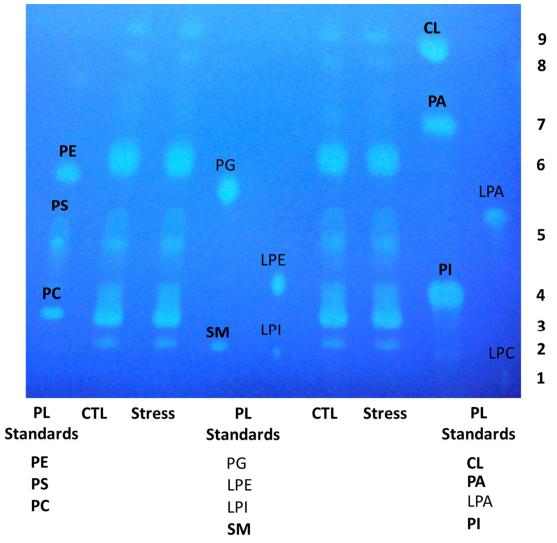
	Myocardium		Brain	
	CTL	Stress	CTL	Stress
Catalase (μmol H_2O_2/min/mg protein)	7.9±0.3	5.9±0.3***	1.3±0.5	2.4±0.2**
Superoxide dismutase (activity Units)	9.1±0.3	8.3±0.8	7.4±0.7	9.3±1.2*
Glutathione reductase (μM NADPH/min/mg protein)	6.6±1.1	7.1±2.3	57.1±13.4	51.2±15.6
GSH/GSSG	15.7±3.2	9.9±2.6*	23.9±4.4	13.4±2.4**

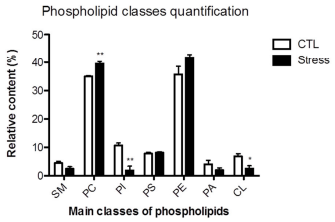
Enzymatic activities in myocardium and brain obtained from mice of control group (CTL) and chronic stress group (Stress) as described in Material and Methods. Values are presented as the mean ±SD of 4-6 independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.005$, values statistically different from controls.

Table 2. Main CL species that decrease after chronic stress and oxidized species identified in CL spectra obtained from mice brain under chronic stress conditions**.**

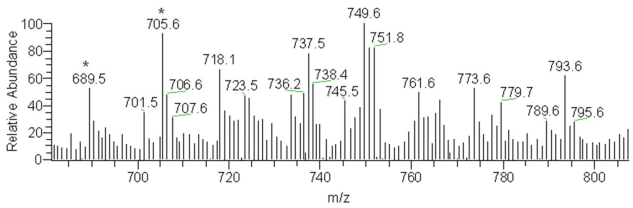
$[M-2H]^{2-}$	**CL species that decrease after chronic stress**
723.4	$(C18:2)_4$ (C18:2)(C18:1)(C20:4)(C16:1) (C18:3)(C18:1)(C20:4)(C16:0)
737.5	$(C18:1)_2$(C22:6)(C16:0) (C18:2)$(C18:1)_2$(C20:4)
749.6	$(C18:1)_2(C20:4)_2$ $(C18:2)_2$ (C18:0)(C22:6) $(C18:1)_2$(C18:2)(C22:6)
751.8	(C18:1)(C18:0)(C20:4)(C20:3) (C18:1)2(C18:0)(C22:6) (C18:1)(C18:2)(C20:3)(C20:2)
	Main CL oxidation products observed after chronic stress
697.8	$(C18:2)_3$(C10:2(O)COOH)
761.7	(C18:1+2O)(C18:1+O)(C22:6)(C16:0)
773.6	(C18:1+2O)(C18:1+O)$(20:4)_2$ (C18:2+2O)(C18:2+O)(18:0)(C22:6) (C18:1)(C18:1+2O)(C18:2+O)(C22:6)



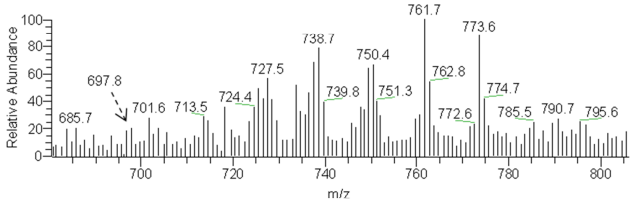




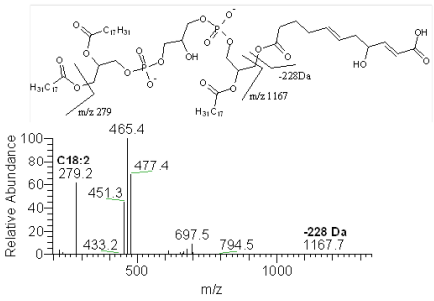
A – Control



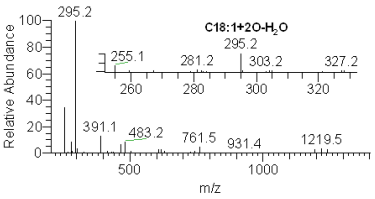
B – Chronic stress



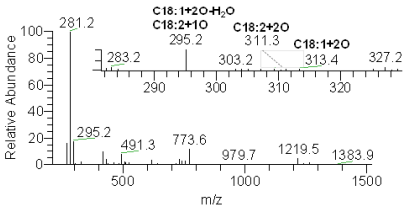
A- MS/MS spectrum of the ion $[M-2H]^{2-}$ at *m/z* 697

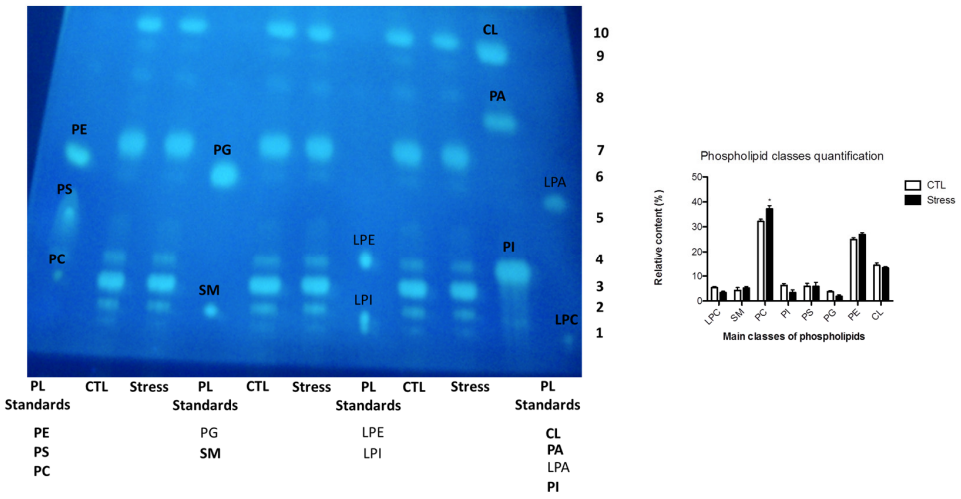


B- MS/MS spectrum of the ion $[M-2H]^{2-}$ at *m/z* 761



C- MS/MS spectrum of the ion $[M-2H]^{2-}$ at *m/z* 773





Highlights

Alterations in phospholipidomic profile in brain of mouse model of depression induced by chronic unpredictable stress

Renata Faria[1], Magda M. Santana[2,3], Célia Aveleira[2], Cláudia Simões[1], Elisabete Maciel[1], Tânia Melo[1], M Manuel Oliveira[4], Francisco Peixoto[5], Pedro Dominggues[1] ,Cláudia Cavadas[2,3], M. Rosário Domingues[1]

[1] Mass Spectrometry Centre, Chemistry Department, University of Aveiro, 3810-193 Aveiro, Portugal
[2]Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal,
[3]Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal
4CQVR, Chemistry Department, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal
5 CITAB, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

*To whom correspondence should be addressed: Maria R. M. Domingues, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.
E-mail: mrd@ua.pt, telephone number: + 351-234-370698, fax number: +351-234-370084

- Depression induced by chronic unpredictable stress have an impact in brain lipidome
- Changes in phospholipidomic profile occurred in brain of a mouse model of depression
- CL oxidation were detected in brain of a mouse model of depression
- Diminished GSH/GSSG corroborates the occurrence of oxidative stress.
- In myocardium only an increase in PC content was disclosed.